UNIVERSITI PUTRA MALAYSIA

MICROSATELLITE DEVELOPMENT AND CROSS SPECIES AMPLIFICATION FOR CHARACTERIZATION OF HATCHERY- BRED *Probarbus jullieni*

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By

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Thesis Submitted to the School of Graduate Studies, Universiti Putra Malaysia, in Fulfilment of the Requirement for the Degree of Master of Science

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Specially dedicated to,
My beloved Father, Mother and my dear sister Somayeh for their invaluable love, understanding, tolerance, sacrifice, moral support

Abstract of thesis presented to the Senate of University Putra Malaysia in fulfilment of the requirement for the degree of Master of Science

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Chairman: Professor Datin Paduka Khatijah Bt Mohd Yusoff, PhD

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Probarbus jullieni is a freshwater ray-finned fish species, found in the rivers of Peninsular Malaysia, it is locally known in Malaysia as Ikan Temoleh. Its natural populations are under serious long-term decline making it to be listed by the International Union for the Conservation of Nature and Natural Resources (IUCN) Red List as 'Endangered' so the conservation status of *P. jullieni* prompted conservationists to plan some conservation programs to rescue this species.

Conservation of genetic resources is an essential component of any species management program and documenting the genetic make up of both natural and farm stocks of the species is an effective strategy to guide management decision for conservation. To this aim, microsatellite markers which have a high degree of polymorphism can be useful in providing valuable information for characterizing populations.

The present study had three major objectives: Detection of genetic variation in hatchery bred *P. jullieni* using microsatellite markers which developed for *P. jullieni* and also cross-amplification of microsatellite primers for closely related species and the second objective is identification and isolation of microsatellite loci in *P. jullieni* and the third one is identification of a sex-specific genomic DNA marker in *P. jullieni* by testing sex-associated markers of other fish species.

Two hatchery populations of *P. jullieni* (Temoleh Siam and Temoleh Tarat) were used in this research. Sixteen microsatellite DNA markers comprising nine MFW and seven SYK primer pairs which were developed for the common carp *Cyprinus carpio L.* and *Tor tambroides*, respectively were tested for cross-amplification of microsatellite loci in the two populations of *P. jullieni*. Thirteen out of these 16 microsatellite primer pairs could detect microsatellites in *P. jullieni* indicating that they can be useful in studying the population structure of the fish species. In addition, six microsatellite DNA markers (Proju primers) which were developed for the seven-line barb (*P. jullieni*) of the Mekong River were used to determine and compare the genetic structure of these two populations of *P. jullieni*.

These 22 primers produced 25 microsatellite loci in Temoleh Siam (ST) and 29 microsatellite loci in Tarat (T) population. The percentage of polymorphic loci was 72% in ST and 56% in T population, the number of observed allele per polymorphic locus ranged from two to six for the ST population and from two to five for the T population. The ST population showed a higher mean effective allele number (1.62) than the T population (1.35). The mean observed heterozygosity (Ho) was higher than the expected heterozygosity (He) in both populations and in

the Siam population it was higher than the Tarat population. The mean F_{IS} in both populations were negative, indicating there is no loss of variability. Eight and three loci showed significant departures from Hardy-Weinberg Equilibrium (HWE) for the ST and T populations, respectively. The analysis of molecular variance (AMOVA) based on 11 polymorphic common loci investigated, showed that 7.31% of the variations were among populations and 92.69% of the variations were within populations. The value of population pairwise F_{ST} in this study was 0.07, which indicated moderate genetic differentiation between these two populations of *P. jullieni*.

In this study ten new microsatellite loci were isolated from *P. jullieni* using a Random Amplified Microsatellites (RAMs) based technique. Five out of ten were polymorphic when tested on *P. jullieni's* individuals.

In order to find a sex-associated marker for estimating sex-ratio in *P. jullieni* populations, two sex-associated primer pairs consisting of a sex specific primer set for medaka (*Oryzias latipes*) and a sex-associated marker for Asian arowana (*Scleropages formosus*) fish, were tested on the male and female samples of *P. jullieni*. The results showed similar banding profile between all the male and female samples. Therefore, these sex-associated DNA markers were unable to detect any differences between the males and females of *P. jullieni* fish.

Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia sebagai memenuhi keperluan untuk ijazah Master Sains

PEMBANGUNAN MIKROSATELIT DAN AMPLIFIKASI SILANG SPESIES UNTUK PENCIRIAN *Probarbus jullieni* YANG-DITERNAK

Oleh

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Pengerusi: Profesor Datin Paduka Dr. Khatijah Mohd Yusoff

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Probarbus jullieni adalah spesies ikan air tawar yang bersirip-rawan terdapat di sungai di Semenanjung Malaysia. Ia dikenali oleh penduduk tempatan sebagai Ikan Temoleh. Populasinya di alam semulajadi semakin brekurangan dan serius dan ini telah menyebabkan ianya disenaraikan di dalam Senarai Merah sebagai 'Terancam' oleh International Union for the Conservation of Nature and Natural Resources (IUCN), maka status pemuliharaan *P. jullieni* ini telah mencetuskan perancangan beberapa program pemuliharaan untuk menyelamatkan spesies ini.

Pemuliharaan sumber genetik adalah komponen penting dalam mana-mana program pengurusan spesies dan mendokumentasikan kandungan genetik bagi stok semulajadi dan ternakan sesuatu spesies merupakan satu strategi yang efektif untuk memimpin keputusan di pihak pengurusan. Untuk mencapai matlamat ini, penanda mikrosatelit yang mempunyai tahap polimorfisme yang tinggi adalah saugat berguna bagi pencirian populasi.

Kajian ini mempunyai tiga objektif utama: Untuk mengesan variasi genetik pada baka ternakan *P. jullieni* dengan menggunakan penand mikrosatelit yang dibangunkan untuk *P. jullieni* dan juga amplifikasi-silang penandan mikrosatelit untuk spesies yang mempunyai perhubungan repat dengannya dan objektif kedua adalah untuk mangenalpasti dan memencilkan penanda mikrosatelit bagi *P. jullieni* dan ketiga adalah untuk mangenalpasti penanda jantina khusus genomik DNA pada *P. jullieni* dengan menguji penanda jantina daripada spesies ikan berlainan.

Dua populasi dari pusat penetasan *P. jullieni* (Temoleh Siam dan Temoleh Tarat) telah digunakan dalam kajian ini. Enam belas penanda DNA mikrosatelit yang mengandungi sembilan pasang primer MFW dan tujuh pasang primer SYK yang telah dibinakan untuk kap biasa, *Cyprinus carpio L.* dan *Tor tambroides*, masing-masing telah diuji untuk amplifikasi-silang lokus mikrosatelit pada kedua-dua populasi *P. jullieni* tersebut. Tiga belas daripada 16 pasang primer ini berjaya mengesan mikrosatelit dalam *P. jullieni*, menunjukkan bahawa mereka boleh digunakan bagi mengkaji struktur populasi *P. jullieni*. Di samping itu, enam penanda mikrosatelit (Proju primers) yang dibangun untuk barb tujuh-garis (*P. jullieni*) dari Sungai Mekong telah digunakan untuk menentukan dan membandingkan struktur genetik pada kedua-dua populasi *P. jullieni* ini.

Dua puluh dua penanda ini telah menghasilkan 25 lokus mikrosatelit untuk Temoleh Siam (ST) dan 29 lokus mikrosatelit untuk populasi Tarat (T). Peratusan lokus polimorfik adalah 72% dalam ST dan 56% dalam populasi T. Jumlah alel diperhatikan per lokus polimorfik berada dalam julat dua hingga enam untuk populasi ST dan dari dua hingga lima untuk populasi T. Populasi ST menunjukkan

nilai purata alel efektif (1.62) berbanding populasi T (1.35). Purata heterozigositi pemerhatian (Ho) adalah lebih tinggi daripada heterozigositi jangkaan (He) pada kedua-dua populasi dan pada populasi Siam lebih tinggi dari populasi T. Purata F_{IS} untuk kedua-dua populasi adalah negatif, menandakan tiada kehilangan kepelbagaian. Sebelas lokus menunjukkan kelainan daripada persamaan Hardy-Weinberg (HWE) masing-masing, pada populasi ST dan T. Analisis varians molekul (AMOVA) berdasarkan pada 11 loci polimorfik lazim yang dikaji menunjukkan bahawa 7.31% varian adalah di kalangan populasi dan 92.69% adalah di dalam populasi. Nilai population Pairwise F dalam kajian ini adalah 0.07, dimana ini menunjukkan kesederhanaan dalam perbezaan genetik antara dua populasi *P. jullieni.*

Dalam kajian ini, sepuluh lokus mikrosatelit baru telah dipencilkan daripada *P. jullieni* melalui kaedah berasaskan Amplifikasi Mikrosatelit Rawak (RAMs). Lima daripada sepuluh adalah polimorfik apabila diuji pada individu *P. Jullieni.*

Untuk mengenalpasti sesuatu penanda jantina bagi menentukan nisbah jantina dalam populasi *P. jullieni*, dua pasang primer berkenaan-jantina yang mengandungi set primer jantina untuk medaka (*Oryzias latipes*) dan penanda jantina untuk ikan arowana Asia (*Scleropages formosos*), telah diuji ke atas sampel jantan dan betina *P. jullieni*. Keputusan menunjukkan profil jalur yang sama antara kesemua sampel jantan dan betina. Maka penanda jantina tersebut gagal untuk membezakan antara jantan dan betina *P. jullieni.*

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This thesis is submitted to the Senate of Universiti Putra Malaysia and has been accepted as fulfilment of requirement for degree of Master of Science .The members of the Supervisory Committee are as follows:

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DECLARATION

I hereby declare that the thesis is based on my original work except for quotations and citations which have been duly acknowledged. I also declare that it has not been previously or concurrently submitted for any other degree at UPM or other institutions.

NAGHMEH GHIASI

Date: 19/6/2009

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LIST OF ABBREVIATIONS

µg	microgram
µl	microlitre
ρmol	picmole
10X	ten times
1X	one time
A	adenosine
bp	base pair
C	cytosine
dATP	deoxyadenosine triphosphate
dCTP	deoxycytidine triphosphate
ddH2O	double distilled water
dGTP	deoxyguanosine triphosphate
DNA	deoxyribonucleic acid
dNTPs	deoxyribonucleotide triphosphate
dTTP	deoxythymidine triphosphate
EDTA	ethylenediamine tetraacetic acid
g	gram
G	guanosine
h	hour
kb	kilobase
kg	kilogram
M	molar
mg	milligram
mg/ml	milligram per millilitre

$MgCl_2$	magnesium chloride
min	minute
mL	millilitre
mM	millimolar
mm	millimetre
ng	nanogram
nm	nanometre
˚C	degree Celsius
OD	optimal density
PCR	polymerase chain reaction
RNA	ribonucleotide acid
s	second
T	thymine
TBE	tris-borate-EDTA buffer
U	unit
UV	ultraviolet
V	volt

CHAPTER1

INTRODUCTION

Species, which are facing a very high risk of extinction in the near future, are considered as endangered, this status prompted conservationist and government agencies to rescue them. *Probarbus jullieni* is a fish species found in the rivers of Malaysia. This has been listed as being endangered in the International Union of the Conservation of Nature and Natural Resources Red Data Book (Poulsen *et al*., 2004). Currently, in Malaysia there are two distinct populations of *P. jullieni,* which have undergone domestication and breeding in the Batu Berendam Station and the Tarat Inland Fisheries hatchery in Sarawak (Bhassu and Zulkafli, 2009).

Genetic diversity is one of the most important factors in the preservation of endangered species. This is due to the effects of genetic diversity on the adaptive flexibility of species to physical and biological environmental changes. The genetic identification and discrimination of a species is a fundamental requirement in any successful conservation program (Reed and Frankham, 2003).

Little is known about the level of genetic variation and population structure of *P. jullieni* (Bhassu and Zulkafli, 2009). Therefore, it is important to evaluate the genetic makeup of *P. jullieni* populations in order to develop breeding policies to achieve better performance. In this respect, genetic markers are effective tools to assess the genetic diversity and population structure. For example, DNA marker-based

polymorphisms can detect higher levels of genetic variations compared to isozymes and morphological markers. Amongst all the different DNA markers, microsatellite markers are currently the most popular tool used to evaluate genetic variability. It is applied widely in endangered species because of the typically high levels of variability detected, coupled with the requirement for only small amounts of tissues. It is a co-dominant nuclear DNA marker inherited in a Mendelian fashion (Leite *et al*., 2007).

DNA markers which are tightly linked to the sex-determination genes provide tools for examining sex linkage aimed to control the sex ratio in breeding programs according to the need. Nevertheless, there is no available report on sex determination of *P. jullieni* using molecular markers.

Therefore in this study the objectives were:

1. To detect genetic variation in hatchery bred *P. jullieni* using microsatellite markers which were developed for *P. jullieni* and also cross-amplification of microsatellite primers developed for closely related species.

2. To identify and isolate new single locus microsatellite markers for *P. jullieni.*

3. To identify a sex-specific genomic DNA marker in *P. jullieni* by testing sex-associated markers of other fish species.

CHAPTER 2

LITERATURE REVIEW

2.1 *Probarbus jullieni*

2.1.1 Taxonomy

According to International Union for the Conservation of Nature and Natural resources (IUCN) 2008 Red List, the classification of "*Probarbus jullieni*" is as follows:

Kingdom:	Animalia
Subkingdom:	Bilateria
Phylum:	Chordata
Subphylum:	Vertebra
Class:	Osteichthyes
Subclass:	Actinoptervgii
Cohort:	Clupeocephala
Order:	Cypriniformes
Family:	Cyprinidae
Genus:	*Probarbus*
Species:	*Probarbus jullieni*

The genus *Probarbus* contains three species *P. labeamajor*, *P. labeaminor* and *P. jullieni. Probarbus jullieni*, has different names, which are Jullien's Golden Carp, Barbeau De Jullien and Seven-stripped Barb. It is locally known in Malaysia as Carpilla Ikan Temoleh and its Fish Base name is Isok barb.

2.1.2 Habitat and distribution

This freshwater ray-finned fish species is distributed in Southeast Asia, Chao Phraya and Mekong basins of Indo-China and Thailand, and the Pahang and Perak basins of Malaysia. It is also found in Cambodia, Laos and Vietnam (Roberts, 1992).

2.1.3 Morphology and biology

P. jullieni is large, attaining 150 cm in length and 70 kg in weight; it lives mainly in the mainstream of large rivers or lakes with moving water, it feeds on aquatic plants, insects and shelled mollusk. Adults and larger juveniles of *P. jullieni* usually have much more red and sometimes yellow coloration on the head, body and fins (Figure 1) than the other two *Probarbus* species. *P. jullieni* is a migratory species. Trophic migrations occur throughout its occurrence range and take place mainly at the onset of the food season and are mainly undertaken by juveniles and sub adults. It has been reported to migrate together with *P. labeamajor* and *P. labeaminor.*

Figure 2.1: Morphology of *P. jullieni* (lateral view)